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Mechanism of Nitrogenase Switch-Off by Oxygen

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Oxygen caused a reversible inhibition (switch-off) of nitrogenase activity in whole cells of four strains of diazotrophs, the facultative anaerobe *Klebsiella pneumoniae* and three strains of photosynthetic bacteria (*Rhodopseudomonas sphaeroides* f. sp. *denitrificans* and *Rhodopseudomonas capsulata* strains AD2 and BK5). In *K. pneumoniae* 50% inhibition of acetylene reduction was attained at an O_2 concentration of 0.37 μM. Cyanide (90 μM), which did not affect acetylene reduction but inhibited whole-cell respiration by 60 to 70%, shifted the O_2 concentration that caused 50% inhibition of nitrogenase activity to 2.9 μM. A mutant strain of *K. pneumoniae*, strain AH11, has a respiration rate that is 65 to 75% higher than that of the wild type, but its nitrogenase activity is similar to wild-type activity. Acetylene reduction by whole cells of this mutant was inhibited 50% by 0.20 μM O_2. Inhibition by CN^- of 40 to 50% of the O_2 uptake in the mutant shifted the O_2 concentration that caused 50% inhibition of nitrogenase to 1.58 μM. Thus, when the respiration rates were lower, higher oxygen concentrations were required to inhibit nitrogenase. Reversible inhibition of nitrogenase activity in vivo was caused under anaerobic conditions by other electron acceptors. Addition of 2 mM sulfite to cell suspensions of *R. capsulata* B10 and *R. sphaeroides* inhibited nitrogenase activity. Nitrite also inhibited acetylene reduction in whole cells of the photodenitrifier *R. sphaeroides* but not in *R. capsulata* B10, which is not capable of enzymatic reduction of NO_2^-. Lower concentrations of NO_2^- were required to inhibit the activity in NO_3^--grown cells, which have higher activities of nitrite reductase. We suggest that the reversible inhibition of nitrogenase activity (switch-off) by oxygen is a particular case of the general phenomenon of diversion of electrons from nitrogenase to other electron acceptors.

Oxygen interferes with biological nitrogen fixation at three different levels. At the genetic level, oxygen acts by repression of nitrogenase synthesis (2, 32, 41). In *Azotobacter chroococcum* oxygen probably also represses synthesis of the putative electron donor, flavodoxin (32). Furthermore, oxygen causes irreversible damage to the enzyme. Dinitrogenase reductases (Fe proteins) are more sensitive to inactivation by O_2 than dinitrogenases (Mo-Fe proteins), but the half-lives of both proteins are a few minutes at the most (33). Finally, oxygen is responsible for reversible inhibition of nitrogenase activity in vivo. Studies on the influence of O_2 on N_2 fixation in *Azotobacter chroococcum* (18, 47), *Derxia gummosa* (17), *Mycobacterium flavum* (4), and *Azospirillum brasilense* (16) have shown that the activity can be inhibited to various degrees by excess oxygenation (switch-off). The activity returns rapidly when aeration is lowered (switch-on) without de novo nitrogenase synthesis (12). These observations led at first to the proposal that O_2 stress causes nitrogenase to assume a conformation in vivo which is inactive, but protected from oxygen (11). Further studies on partially purified nitrogenase preparations from *Azotobacter vinelandii* (15) and *Azotobacter chroococcum* (31) have suggested that this conformational protection results from association of the enzyme with a third protein. In both cases it was shown that nitrogenase proteins formed an O_2-stable complex with nonheme iron proteins.

In a previous paper (20) it was shown that molecular oxygen also causes a reversible inhibition of nitrogenase activity in vivo in three strains of photosynthetic bacteria. This switch-off was either partial or complete, with 50% inhibition occurring at O_2 concentrations of 0.73 μM for *Rhodopseudomonas capsulata* B10, 0.32 μM for *Rhodospirillum rubrum*, and 0.26 μM for *Chromatium vinosum*. When respiration of *Rhodopseudomonas capsulata* was inhibited with cyanide, 50% inhibition of acetylene reduction was shifted to 2.03 μM O_2. In this paper we show that the switch-off of nitrogenase activity is a general phenomenon. In accordance with previous findings we suggest that O_2 causes inhibition by competing for a limited supply of reducing power. We show that the degree of the switch-off can be affected by an increase or decrease in respiration in the presence of oxygen. Most significantly, the phenomenon does not depend exclusively on the presence of oxygen; under anaerobic conditions, either nitrite or sulfite can act equally well as an effector of the switch-off.

MATERIALS AND METHODS

Organisms and culture conditions. *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* IL 106 was a gift from T. Satoh, Department of Biology, Faculty of Science, Tokyo Metropolitan University, Tokyo, Japan. *Rhodopseudomonas capsulata* AD2 and BK5 were gifts from J. H. Klemme, Institut für Mikrobiologie, Rheinische Friedrick-Wilhelm-Universitat, Bonn, Federal Republic of Germany. *Klebsiella pneumoniae* KP1 was a gift from A. Zamir, Department of Biochemistry, Weitzman Institute of Science, Rehovot, Israel. All strains of *Rhodopseudomonas capsulata* were grown anaerobically in the light at 30°C on the medium described by Ormerod et al. (28) with 20 mM glutamate and 30 mM lactate as the nitrogen and carbon sources, respectively; this medium was supplemented with 0.002% thiamine hydrochloride. *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* was grown anaerobically in the light at 30°C on a medium described by Sawada et al. (36) with 20 mM glutamate and 30 mM malate as the nitrogen and carbon sources, respectively. *K. pneumoniae* was grown under anaerobic conditions on NFDM medium (8) with 5% glucose as the carbon source and was derepressed for nitrogenase during a period of 20 h with N_2 as the sole

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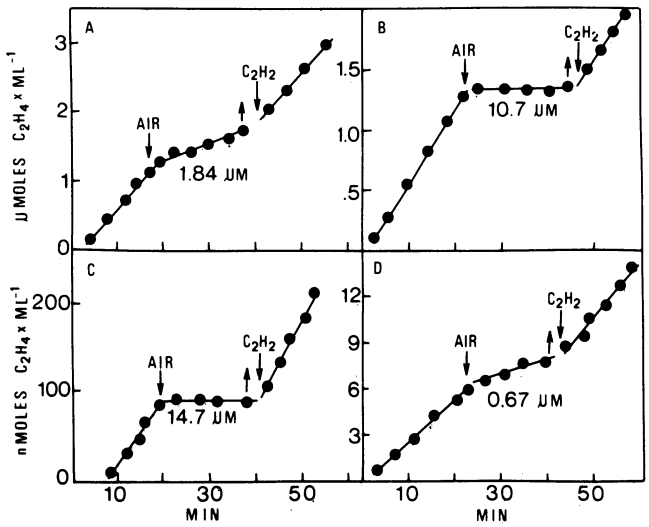

FIG. 1. Effect of O_2 on acetylene reduction activity of whole cells. The assays were run in the apparatus described in reference 20. (A) *Rhodopseudomonas sphaeroides* f. sp. *denitrificans*. (B) *Rhodopseudomonas capsulata* BK5. (C) *Rhodopseudomonas capsulata* AD2. (D) *K. pneumoniae*. The left-hand part of each graph shows the control rate of acetylene reduction assayed under anaerobic conditions (argon in panels A through C and nitrogen in panel D). The arrows labeled AIR and C_2H_2 indicate when gases were added, and the numbers show the steady-state concentrations of O_2 in the cell suspensions. The unlabeled arrows indicate the times of sparging with argon or nitrogen (according to the control conditions). The right-hand part of each graph represents the activity after anaerobic conditions were restored.

nitrogen source. *K. pneumoniae* mutant strain AH11 was obtained fortuitously when we were selecting for high-catalase mutants by a series of transfers of an aerobic culture in hydrogen peroxide-containing media.

Enzymatic activities. Nitrogenase activity was assayed by the acetylene reduction method. Anaerobic acetylene reduction in whole cells and in vivo assays in the presence of oxygen were performed with the apparatus described by Hochman and Burris (20) which enables simultaneous measurement of acetylene reduction and dissolved oxygen concentration. All assays with photosynthetic bacteria were run under saturated light conditions (200 microeinsteins m^{-2} s^{-1}). Crude extracts were prepared anaerobically by passing the cells through a French pressure cell at 18,000 lb/in^2. The extract was centrifuged for 10 min at 15,000 × *g* to remove unbroken cells and then for 60 min at 140,000 × *g*. The supernatant was used for in vitro nitrogenase assays, and the chromatophores in the pellet were suspended in 100 mM Tricine [*N*-tris(hydroxymethyl)methylglycine buffer], pH 7.5 (Sigma Chemical Co., St. Louis, Mo.). Acetylene reduction in the crude extract was measured as described previously (20). Photophosphorylation was measured in the chromatophore fraction with ^{32}P, as described by Hochman et al. (21). Respiration was measured at 30°C by monitoring O_2 consumption with a Clark type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio).

Other methods. Bacteriochlorophyll concentrations were measured in an acetone-methanol extract by using the method of Clayton (10). Protein was determined by the method of Lowry et al. (23). For total cell protein measurements the pelleted cells were boiled for 20 min in 1 N NaOH, and the supernatant after centrifugation was used for the assay.

Chemicals. Acetylene was generated from calcium carbide. ADP, ATP, creatine kinase (EC 2.7.3.2), and phosphocreatine were purchased from Sigma Chemical Co. Sodium dithionite was obtained from E. Merck AG. All other chemicals were of analytical grade.

RESULTS

Switch-off of nitrogenase by oxygen. Addition of oxygen to anaerobic cultures caused inhibition of acetylene reduction activity in the photosynthetic bacteria *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* (Fig. 1A) and *Rhodopseudomonas capsulata* BK5 (Fig. 1B) and AD2 (Fig. 1C) and also in *K. pneumoniae* (Fig. 1D). In all cases, depending on the O_2 concentration and duration of the treatment, all or a great part of the original activity was recovered after removal of the oxygen.

Effect of respiration rates on the degree of O_2 switch-off. In *K. pneumoniae*, 0.37 μM O_2 caused 50% inhibition of C_2H_2 reduction (Fig. 2), whereas 5.3 μM O_2 inhibited C_2H_2 reduction completely. At concentrations of O_2 up to 1 μM, full activity was recovered after restoration of anaerobic conditions; at higher oxygen concentrations (up to 5.3 μM O_2) only 80 to 90% of the activity was recovered (data not shown). A cyanide concentration of 90 μM inhibited respiration by 60 to 70% in whole cells of *K. pneumoniae* (Table 1) but did not affect nitrogenase activity. Addition of O_2 in the presence of 90 μM cyanide to cell suspensions of *K. pneumoniae* caused the same effect of reversible inhibition of nitrogenase activity as observed in the absence of the inhibitor (data not shown). However, a plot of acetylene reduction activity versus dissolved O_2 concentration in the presence of cyanide (Fig. 2) revealed that for each O_2 concentration the activity was higher in the presence of the inhibitor than in the absence of the inhibitor; 2.9 μM O_2 was required for 50% inhibition of the activity when cyanide was present.

A mutant strain of *K. pneumoniae*, strain AH11, which

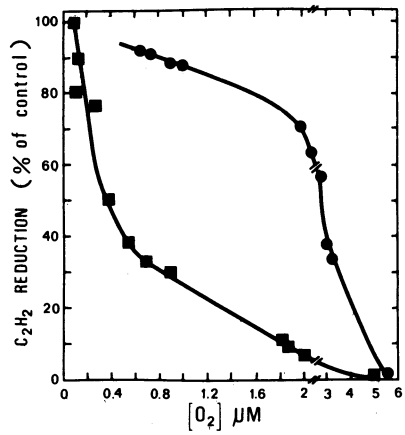

FIG. 2. Effect of dissolved O_2 concentrations on the acetylene reduction activity of whole cells of *K. pneumoniae*. The values were calculated from experiments run under the same conditions as those described in the legend to Fig. 1D. The control rate of 100% was the value obtained for each assay before treatment with O_2. Symbols: ■, activity in the presence of O_2; ●, activity in the presence of O_2 and 90 μM CN^-. The experimental conditions used are described in Materials and Methods.

TABLE 1. Whole-cell activities of nitrogenase and respiration in wild-type *K. pneumoniae* and *K. pneumoniae* AH11[a]

Strain	Cyanide concn (μM)	Nitrogenase activity[b]	Respiration[c]
Wild type	0	175 ± 26.5	787 ± 61
	90	175 ± 26.5	291 ± 15
AH11	0	180 ± 27	1,345 ± 121
	70	180 ± 27	1,022 ± 59

[a] Cells were grown anaerobically with N_2 as the sole nitrogen source. The rest of the experimental details are described in Materials and Methods.
[b] Nanomoles of C_2H_4 formed per milligram of protein per hour.
[c] Nanomoles of O_2 consumed per milligram of protein per hour.

had respiration rates that were 65 to 75% higher than those of the wild type but similar nitrogenase activities, was also investigated (Table 1). Assays of the effect of oxygen on nitrogenase activity in whole cells of AH11 showed that at each O_2 concentration the activity was lower in the mutant than in the wild type, with 50% inhibition occurring at a concentration of 0.20 μM (data not shown); 70 μM cyanide inhibited 40 to 50% of the whole-cell respiration but did not affect the acetylene reduction rates (Table 1). Inhibition of acetylene reduction by oxygen in AH11 was always lower in the presence of cyanide than in its absence (data not shown), and 1.58 μM O_2 caused 50% inhibition of the activity. These findings are in accordance with our working hypothesis that the switch-off of acetylene reduction activity in the presence of oxygen is caused by a shortage of electrons available for nitrogenase activity, when electrons are diverted to support respiration (electron diversion hypothesis).

Switch-off of nitrogenase activity under anaerobic conditions. The evidence presented above suggests that the switch-off of acetylene reduction activity in the presence of oxygen was caused by a shortage of electrons available for nitrogenase activity, because of their diversion to support respiration. According to this hypothesis, the same phenomenon should occur anaerobically in the presence of other metabolites which are reduced by the cells. To test this hypothesis, we used sulfite (SO_3^-) and nitrite (NO_2^-). Sulfite is reduced to sulfide with the enzyme sulfite reductase (37). Figure 3 shows that addition of sulfite to cell suspensions of *Rhodopseudomonas capsulata* (Fig. 3A) and *Rhodopseudomonas sphaeroides* (Fig. 3B) caused inhibition of acetylene reduction activity. Nitrite can be reduced by the enzyme nitrite reductase. This enzyme is present in *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* (36) but not in *Rhodopseudomonas capsulata* B10 (9). In *Rhodopseudomonas sphaeroides* nitrite reductase activity is significantly higher in cells grown in the presence of nitrate (26). Figure 4 shows that, after the addition of NO_2^- to *Rhodopseudomonas sphaeroides* cells, acetylene reduction activity was inhibited, but there was a difference between cells grown with nitrate and cells grown without nitrate. In cells grown in the presence of NO_3^-, 0.1 and 0.05 mM NO_2^- caused complete inhibition of the activity (Fig. 4A). In the case of 0.05 mM NO_2^- the inhibition was relieved after NO_2^- was exhausted. When the cells were grown without nitrate, acetylene reduction activity in vivo was inhibited with 0.25 mM NO_2^- but not with lower concentrations (Fig.

TABLE 2. Effect of sulfite and nitrite on nitrogenase and photophosphorylation activities in *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* and on photophosphorylation activity in *Rhodopseudomonas capsulata* B10[a]

Organism	Addition	Nitrogenase activity[b]	Photophosphorylation activity[c]
Rhodopseudomonas capsulata	None		77
	2 mM SO_3^-		83
	0.1 mM NO_2^-		75
	1 mM NO_2^-		76
Rhodopseudomonas sphaeroides	None	0.50	23
	2 mM SO_3^-	0.49	27
	0.1 mM NO_2^-	0.50	21
	1 mM NO_2^-	0.49	22

[a] Acetylene reduction was assayed in crude extracts, and photophosphorylation was assayed in chromatophore fractions as described in Materials and Methods.
[b] Nanomoles of C_2H_4 formed per milligram of protein per hour.
[c] Nanomoles of O_2 consumed per milligram of protein per hour.

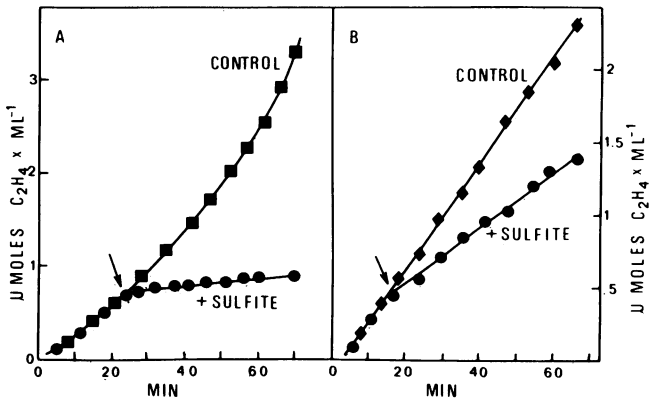

FIG. 3. Effect of 2 mM sodium sulfite on the acetylene reduction activity of whole cells of *Rhodopseudomonas capsulata* (A) and *Rhodopseudomonas sphaeroides* (B). The arrows indicate when 20 μl of an anaerobic solution of Na_2SO_3 was added. Experimental details are described in Materials and Methods.

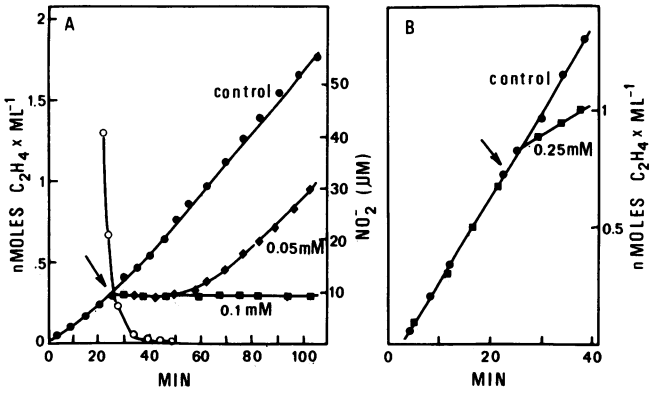

FIG. 4. Effect of nitrite on the acetylene reduction activity of whole cells of *Rhodopseudomonas sphaeroides*. The arrows indicate when an anaerobic solution of KNO_2 was added, and the graphs show the initial nitrite concentrations in the cell suspensions. (A) Culture grown in the presence of 10 mM KNO_3. Cells were centrifuged, washed, and suspended in nitrate-free medium under anaerobic conditions prior to the assay. Symbols: ●, control; ◆, 0.05 mM KNO_2; ■, 0.1 mM KNO_2; ○, NO_2 concentration in the cell suspension. (B) Culture grown in the absence of nitrate. Symbols: ●, control; ■, 0.25 mM KNO_2. The rest of the experimental details are described in Materials and Methods.

4B). Addition of 0.5 mM NO_2^- to *Rhodopseudomonas capsulata* B10 cells did not effect C_2H_2 reduction activity (data not shown).

Both sulfite and nitrite represent intermediate oxidation states which can undergo oxidation and reduction and thus might interfere with enzymatic activities. Therefore, we checked for a possible direct effect of these ions on nitrogenase or on the production of ATP by photophosphorylation. Table 2 shows that SO_3^- at the same concentration that inhibited nitrogenase activity in whole cells did not inhibit acetylene reduction in crude extracts of *Rhodopseudomonas capsulata* and *Rhodopseudomonas sphaeroides* and somewhat enhanced photophosphorylation activity in both strains. Nitrite at concentrations up to 1 mM did not affect photophosphorylation activity in chromatophores from either *Rhodopseudomonas capsulata* or *Rhodopseudomonas sphaeroides*. Nitrogenase activity was not inhibited by nitrite concentrations up to 1 mM.

DISCUSSION

Numerous studies of various aspects of biological nitrogen fixation have shown that this is a conserved process. There are marked similarities among a wide range of microorganisms in the physiological and biochemical aspects of nitrogen fixation (3, 6, 7, 13, 14, 18, 21, 24, 25, 27, 39, 47). The data presented in this paper, together with previously published similar findings (18, 20, 47), show that the reversible inhibition of acetylene reduction activity in vivo by oxygen, termed switch-off, is an inherent property of nitrogen fixation that should be added to the list of similarities among N_2-fixing microorganisms.

Two different explanations are currently offered to account, on the one hand, for the inhibition of the enzymatic activity in the presence of oxygen and, on the other hand, for the lack of irreversible damage to the enzyme. The first explanation was proposed when the reversible inhibition of nitrogenase activity was observed in *Azotobacter chroococcum* and is related to a protection mechanism that is called conformational protection (11). Robson (32) isolated from this organism an O_2-tolerant nitrogenase complex which contained a 2Fe-2S protein and associated it with the mechanism of this conformational protection. Haaker and Veeger (15) and Scherings et al. (38) showed in *Azotobacter vinelandii* that a 2Fe-2S protein, which was first isolated by Shethna et al. (40), could be added to nitrogenase to form an oxygen-stable complex, which these investigators interpreted as representing the switched-off enzyme. This complex could be isolated only when the three proteins were in the oxidized state and $MgCl_2$ was present. These binding studies are important in relation to the protein chemistry of nitrogenase, but their physiological relevance to the switch-off phenomenon and protection against oxygen damage has not been clearly demonstrated yet, both because of lack of specificity, since nitrogenase proteins have been shown to bind various cell components (5, 22, 45, 46), and because of the finding that the half-life for autooxidation of the reduced *Azotobacter chroococcum* protective protein is 4.5 min (31), whereas the switch-off is instantaneous.

An alternative explanation for the switch-off of nitrogenase is that molecular oxygen competes with dinitrogen for electrons. Although this explanation was suggested in several studies (20, 29, 30, 47), the first direct experimental evidence was given by Hochman and Burris (20). It was shown that partial inhibition of respiration in *Rhodopseudomonas capsulata* caused an increase in the O_2 concentration required for the switch-off of nitrogenase. In this paper we extend this finding to other N_2-fixing microorganisms and present more experimental data in support of this theory. Our results concerning the effective oxygen concentrations which cause inhibition of nitrogenase in whole cells of *K. pneumoniae* (50% inhibition at a concentration of 0.37 μM and total inhibition at concentrations higher than 5.3 μM) are in agreement with the findings of Hill et al. (19). These investigators measured the influence of oxygen on nitrogenase activity by using a technique which exploits leghemoglobin to measure the dissolved O_2 concentrations. They showed that 50% inhibition, relative to anaerobic conditions, was attained at 0.25 to 0.40 μM O_2 and that the activity approached zero at 0.55 μM O_2. However, Hill et al. made no attempt to explain the nature of the inhibition. In another study Bergersen et al. (1) showed that the K_s of the dominant terminal oxidase in *K. pneumoniae* was 0.08 μM O_2. In agreement with this finding our data show that oxygen starts to inhibit nitrogenase activity at a concentration of about 0.1 μM, which indicates that it is the activation of respiration that causes the inhibition. Furthermore, in the mutant AH11, which has higher respiration rates, acetylene reduction activity is lower than acetylene reduction activity in the wild type at each O_2 concentration. In accordance with what was shown in *Rhodopseudomonas capsulata* (20), partial inhibition of respiration by CN^- caused an increase in acetylene reduction both in the wild type and in the mutant AH11 at each O_2 concentration. These results indicate that, at low concentrations of dissolved O_2 in the culture, respiration is capable of scavenging oxygen fast enough so that the interior of the cells is kept anaerobic, and this accounts for the lack of irreversible damage to the enzyme. On the other hand, at this range of oxygen concentrations, respiratory activity increases with the increase in the dissolved O_2 concentrations. This in turn causes the diversion of increasing proportions of electrons to the reduction of oxygen, thereby creating a shortage of electrons available for acetylene reduction. In *Klebsiella aerogenes* it has been shown (44) that addition of oxygen to anaerobic cultures causes a fast decrease in the ratio of NADH to NAD^+. If a similar response occurs in nitrogen-fixing cells, it could also affect the ratio of ferredoxin reduction to ferredoxin oxidation, which might result in a lower steady-state concentration of reduced ferredoxin and, hence, slower electron transport to nitrogenase.

Our results on the reversible inhibition of nitrogenase by oxygen indicate that this switch-off phenomenon is not a mechanism of protection against oxygen damage. Rather, it is a consequence of the system in which an electron acceptor competes with other enzymes, whose activities are dependent on a common pool of reducing power. In keeping with this hypothesis are our results on the switch-off in vivo of acetylene reduction under anaerobic conditions by sulfite and nitrite. Both of these substrates are reduced by the corresponding reductases that derive their reducing power from the photosynthetic electron transport chain (34, 37), which is also the source of reduced ferredoxin utilized by nitrogenase (27). Indeed, the switch-off by nitrite is correlated with the presence of nitrite reductase activity and its level. Sulfite and nitrite have been shown to inhibit nitrogenase in vitro (42, 43); however, the range of concentrations used in our study does not affect acetylene reduction in crude extracts from either *Rhodopseudomonas capsulata* or *Rhodopseudomonas sphaeroides* (Table 2). Satoh and Shimazaki (35) have shown that both nitrate and nitrite inhibit acetylene reduction activity in whole cells of *Rhodo-*

pseudomonas sphaeroides f. sp. *denitrificans*, with nitrite having a more pronounced effect and exerting its inhibitory effect in cells grown without nitrate. However, these investigators used much higher concentrations of the inhibitor (0.5 to 10 mM) than we did (0.05 to 0.25 mM).

In summary, we demonstrate in this paper, in agreement with previous results (20), that the switch-off of nitrogenase activity in vivo by oxygen in phototrophs and in *K. pneumoniae* is a natural consequence of the diversion of electrons from nitrogenase to oxygen.

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